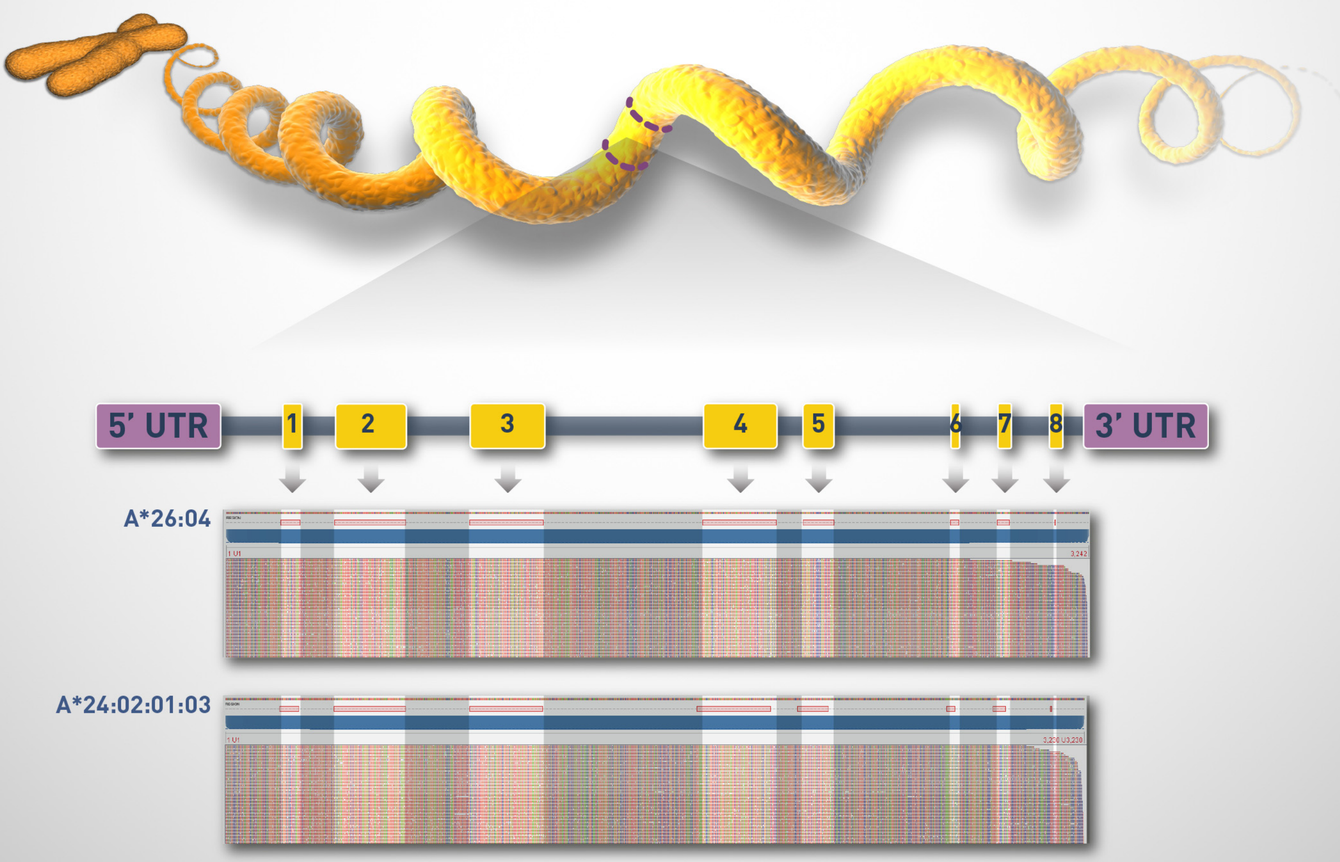
HLA Getting Started Guide



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Pacific Biosciences of California, Inc.
1380 Willow Road
Menlo Park, CA 94025
www.pacificbiosciences.com

HLA Amplicon Sequencing

The human leukocyte antigen (HLA) is the human version of the major histocompatibility complex (MHC) genes found in most vertebrates. This group of genes resides on Chromosome 6 (see Figure 1) and codes for proteins that regulate the human immune system. Some of these proteins are denoted as antigens, due to their discovery as key factors in organ transplant tolerance. Different classes of HLA genes are essential elements for immune function. However, only specific HLA genes corresponding to MHC Class I (HLA A, B, and C) and MHC Class II (HLA DP, DQ, and DR) present antigens that are known to have a major role in causing organ transplant rejections and providing protection mechanisms in some cancers. Additionally, these antigens are known to show a linkage-based association with autoimmune diseases including type I diabetes, celiac disease, and others.

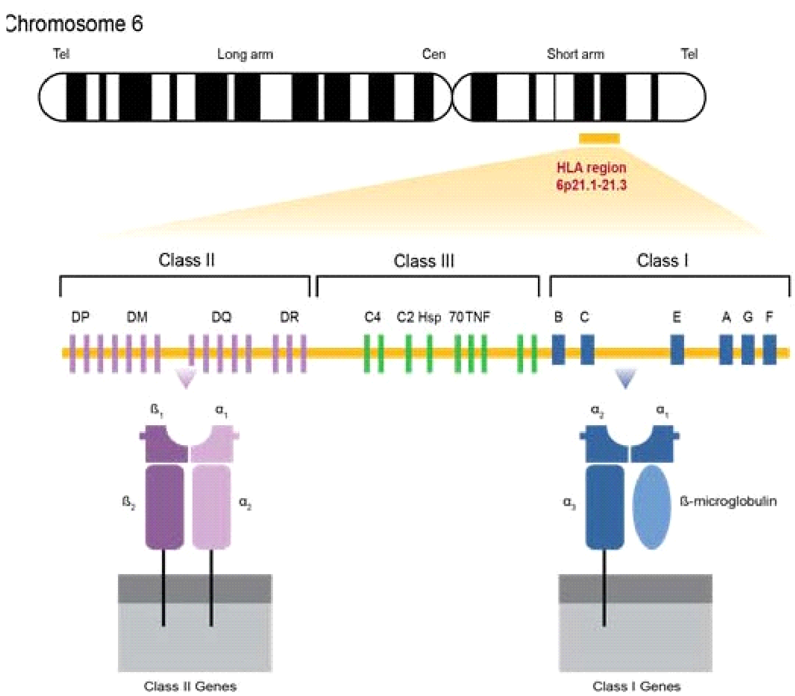

Figure 1 HLA Genes on Chromosome 6

The HLA genes are among the most polymorphic genes in the human genome. Therefore, it is very difficult to find two unrelated individuals with identical HLA molecules. As a result, HLA Class I and Class II genes are routinely sequenced or stereotyped with well-established methods for research in tissue transplantation, autoimmune disease-association studies, drug hypersensitivity research, and other applications.

This guide describes recommended protocols for sequencing HLA Class I and Class II genes and for producing fully-phased, unambiguous, allele-level information on the PacBio® System. An end-to-end HLA sequencing workflow, using SMRT® technology and third-party tools, is provided along with various strategies for effective multiplexing.

HLA genes can be amplified using homebrew assays or commercial PCR reagent kits (e.g., NGS-go® reagents from GenDx). Depending on the target region, full-length genes (Figure 2) or partial amplicons (Figure 3) ranging from ~1.5 kb to 11 kb can be directly converted into SMRTbell™ libraries without additional shearing.

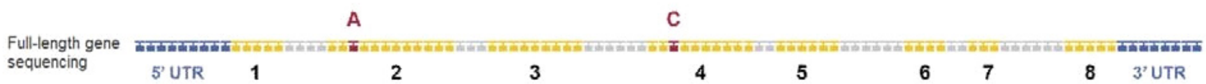

Figure 2   Fully Phased Allele-Level Sequencing

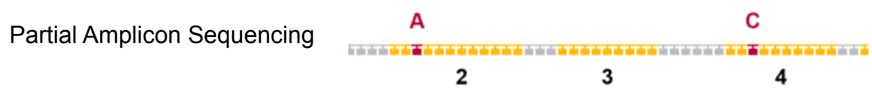

Figure 3   SNP Detection and Phasing in Exons 2, 3, and 4 Only

HLA Amplicon Sequencing

Sequencing on the PacBio System generates long reads (>3 kb) that allow resolution of polymorphism across the HLA target amplicons along with phasing. Complete information can be obtained from a span of a single read. Therefore, PCR amplicons (of targeted regions) can be amplified and sequenced in their entirety with no assembly required.

Consensus sequences generated from the full-length reads leave no ambiguity about phasing between SNPs or any other types of variations that might be present along the length of the targeted region. PacBio's SMRT technology is an optimal solution for allele-level HLA typing (See Figure 4).

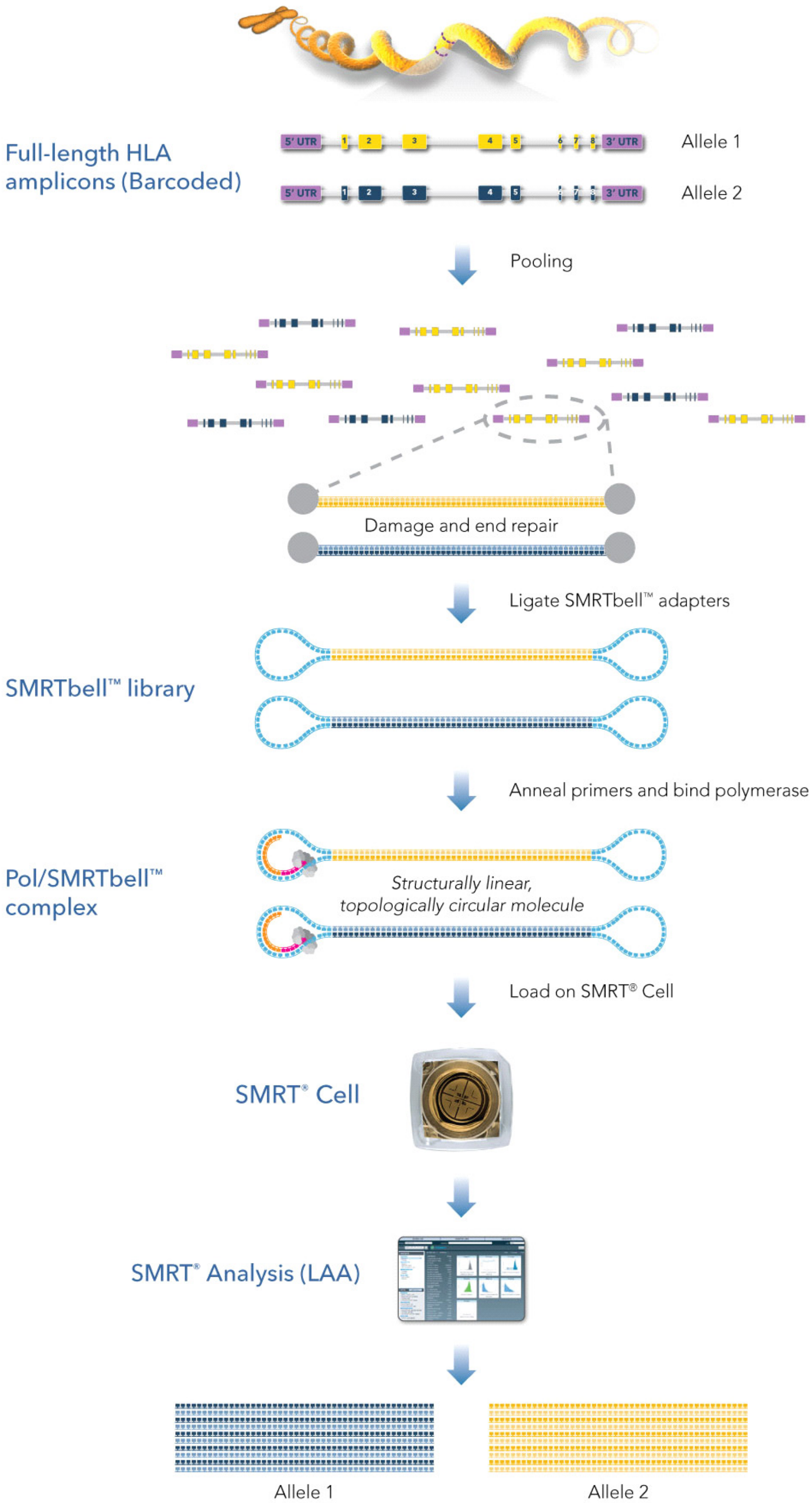

Figure 4 HLA Amplicon Sequencing. Whole HLA genes are amplified and sequenced in their entirety with no assembly required, allowing analysis of both alleles and phasing.

Customer-Provided and Commercially Available Assays

PacBio protocols and reagents provides researchers the flexibility to use commercial off-the-shelf assays or design and adopt customer provided reagents for targeted sequencing of full-length or relevant-region HLA amplicon sequencing on Single Molecule, Real-Time (SMRT®) Sequencing platforms. Using SMRT technology, high-quality consensus sequences (QV 50 or > 99.999% accuracy) can be obtained from contiguous reads that span complete amplicons up to 10 kb in length. This ultimately allows imputation-free typing of the HLA genes.

CE markerd commercial assays for HLA typing from GenDx are fully validated on the PacBio RS II platform. The NGS-go (GenDx) kit provides fully optimized primer sets for amplifying full-length highly polymorphic full-length HLA class I genes and relevant regions of Class II genes including the peptide binding domains. Learn more about NGS-go kits at www.gendx.com.

Multiplexing HLA Amplicons

Cost-efficient multiplexed amplicon sequencing can also be adopted with the PacBio RS II platform using PacBio's specific barcoding system. Non-overlapping amplicons may be multiplexed and sequenced in a single SMRT Cell. For example, non-overlapping amplicons from a single patient maybe pooled without the need for barcoding.

As your throughput increases, the cost per sample can be reduced further by integrating barcoding into the workflow. This allows pooling of amplicons from multiple patients to be sequenced in one SMRT Cell. To maximize SMRT Cell capacity, the following three barcoding strategies may be used:

1. Barcoded Locus-Specific Primers (Figure 5)
2. Barcoded Universal Primers (highly recommended; Figure 6)
3. Barcoded Adapters (highly recommended; Figure 7)

All workflows include barcodes to uniquely identify samples, but they differ in how the barcodes are incorporated into the SMRTbell template.

For the locus-specific barcoding primer approach, Excel files for ordering primers with the recommended barcodes can be found in the links below. A set of 384 16-bp barcodes are designed for SMRT Sequencing.

http://www.pacb.com/wp-content/uploads/PacBio-PCR-Primer-Barcodes-0001-to-0096-IDT-Template.xlsx

http://www.pacb.com/wp-content/uploads/PacBio-PCR-Primer-Barcodes-0097-to-0192-IDT-Template.xlsx

http://www.pacb.com/wp-content/uploads/PacBio-PCR-Primer-Barcodes-0193-to-0288-IDT-Template.xlsx

http://www.pacb.com/wp-content/uploads/PacBio-PCR-Primer-Barcodes-0289-to-0384-IDT-Template.xlsx

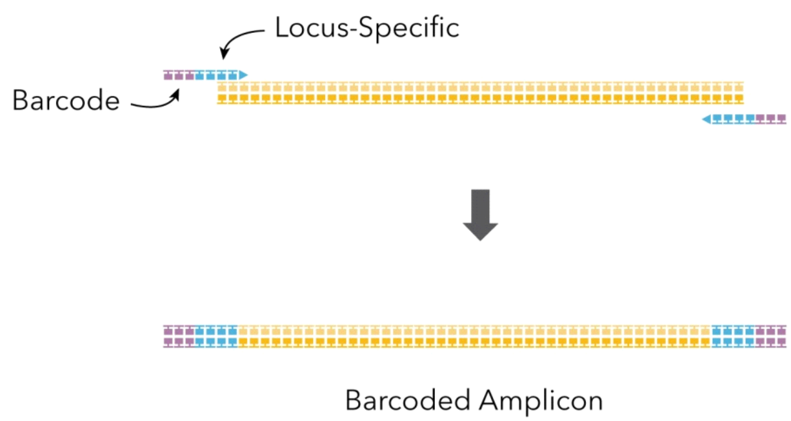

Figure 5 Locus-specific primers may be barcoded using published PacBio 16-bp barcodes.

The Barcoded Universal Primer approach allows for more flexibility in primer design, modification and optimization. In this method, the barcode is incorporated into the PCR amplicon via a two-step tailed PCR approach. As shown in Figure 6, internal primers that are a combination of universal and target-specific sequences are used for PCR1. The universal sequences are incorporated into the PCR amplicon after the first amplification step. In the second round of PCR (PCR2), the barcoded universal primers (supplied by PacBio) are used for the amplification in order to incorporate the barcodes.

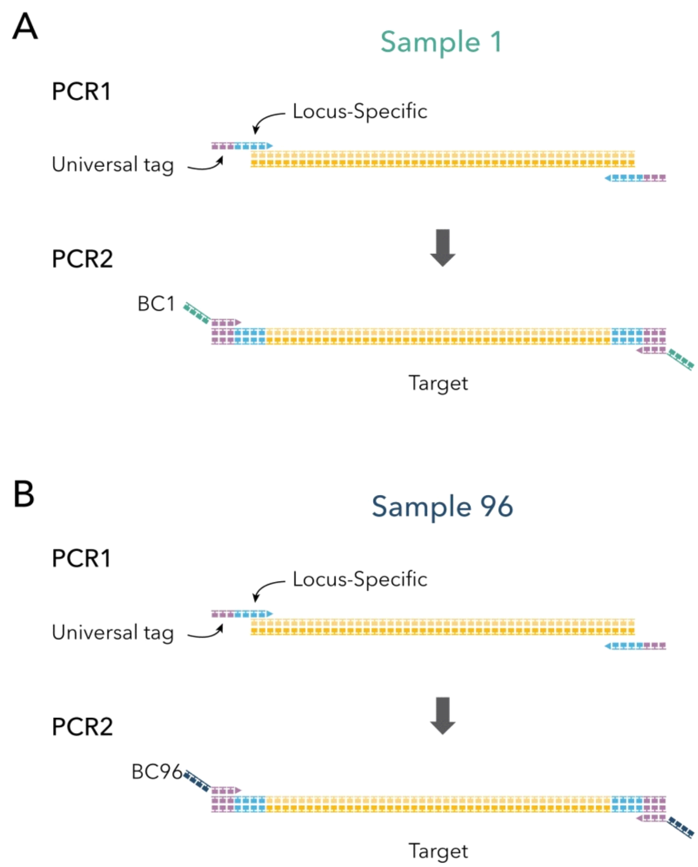

Figure 6 Barcoding using Barcoded Universal Primer

The third multiplexing solution adds the barcode to the SMRTbell adapter, as shown in Figure 7. In this method, the barcode is introduced to each amplicon through ligation during SMRTbell library preparation. Similar to Barcoded Universal Primers, Barcoded Adapters are commercially available and may be purchased from PacBio.

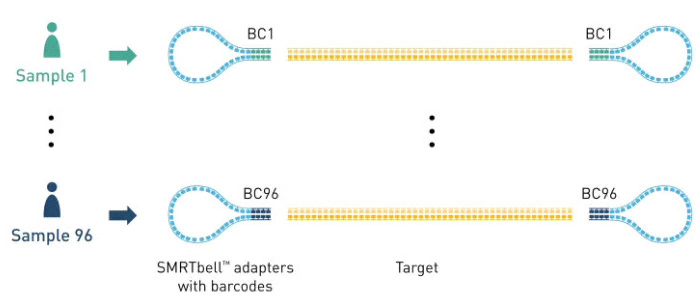

Figure 7 Barcoding using Barcoded Adapter

The procedure combines end-repair and barcoded adaptor-ligation into a single step. Once samples are barcoded, they can be pooled for DNA damage repair, purification and sequencing in one SMRT Cell, thus reducing the overall library cost. The Barcoded Adapter workflow is particularly recommended when primer sets are already fully validated and an existing workflow is preferred.

Recommended Coverage per Amplicon:

When pooling samples, it is imperative to take into account the required coverage needed per amplicon. PacBio recommends >100x subreads per amplicon. However, a higher coverage is preferred. Based on this coverage requirement, you can estimate the number of samples to pool and the number of SMRT Cells necessary to achieve good coverage.

For example, Class I, GenDx, ~3.3 kb, assumptions:

Poisson distribution loading of 35%

For 3.3 kb amplicons, average 2-3 subreads, 4-hr movie

500x coverage per amplicon

- Total available zero-mode waveguides are 150,000
- 150,000 ZMWs X 35% = 52,500 reads
- ~2-3 subreads per read = >105,000
- 105,000 subreads x 0.80 (barcoded yield) = 84,000 subreads
- 84,000 / 500x coverage = ~168 Amplicons with 500x coverage from 1 SMRT Cell

Designing HLA Pools

PacBio highly recommends performing small-scale and controlled experiments to test performance of the system and understand the required coverage per amplicon.

Option 1: Same Size Amplicons
Pool amplicons of similar size, generally within 10% the size of each other. For example, Class I genes (HLA-A, HLA-B and HLA-C) amplified using the NGS-go primers generate amplicons approximately 3.2 to 3.5 kb in size (see Figure 8) that can be pooled for SMRT Sequencing.

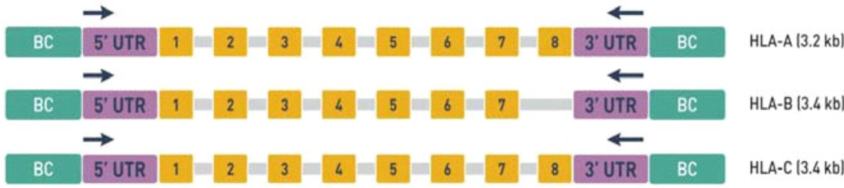

Figure 8   Pooling of HLA Class I Genes. Using the NGS-go assay, Class I genes have similar amplicon sizes that can be multiplexed and sequenced to generate full-length HLA genes (~3.2 to 3.5 kb HLA Class I genes).

Same size amplicons may be pooled using an equal-mass approach to minimize allelic imbalance as a result of imbalanced pooling. For best practice, pooling equimolar amounts of each amplicon is desired. Quantify amplicons using a system that provides both size and concentration information (Agilent BioAnalyzer system). The size and concentration information is necessary for calculating the amount of each sample for equimolar pooling. An excel-based Pooling Calculator is available on our website (www.pacb.com/wp-content/uploads/HLA-Pooling-Calculator.xlsx). Use the above recommendation when first evaluating HLA typing performance by SMRT Sequencing.

As throughput increases, you may decide to pool samples by equal-volume instead of equal-mass. This method works only if your PCR reactions generate consistent yields across all samples.To QC PCR products, running samples on by agarose gel electrophoresis may be a more convenient option (see Figure 9).

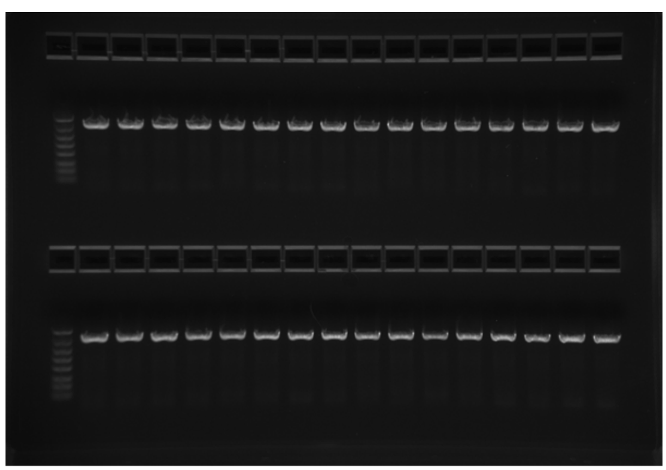

Figure 9 HLA A amplicons loaded on agarose gel (1.2% Lonza® FlashGel). If the yield across all samples is consistent, samples may be pooled by equal-volume.

For low-performing amplicons (weak signal), higher volume may be necessary for pooling. If low-performing amplicons show insufficient coverage, they may be re-pooled and re-processed for additional coverage.

Option II: HLA Class I and Class II Mixed Amplicon Pools

While pooling amplicons of same size is highly recommended, HLA Class I and Class II genes of varying sizes (Figure 10) may also be pooled. This is recommended only if your throughput is low. In SMRT Sequencing, small-insert SMRTbell templates are preferentially loaded over large-insert SMRTbell templates thus resulting in biased coverage. To minimize bias, mixed-size amplicons should be pooled in equal-molar amounts using the calculator provided ((www.pacb.com/wp-content/uploads/HLA-Pooling-Calculator.xlsx).

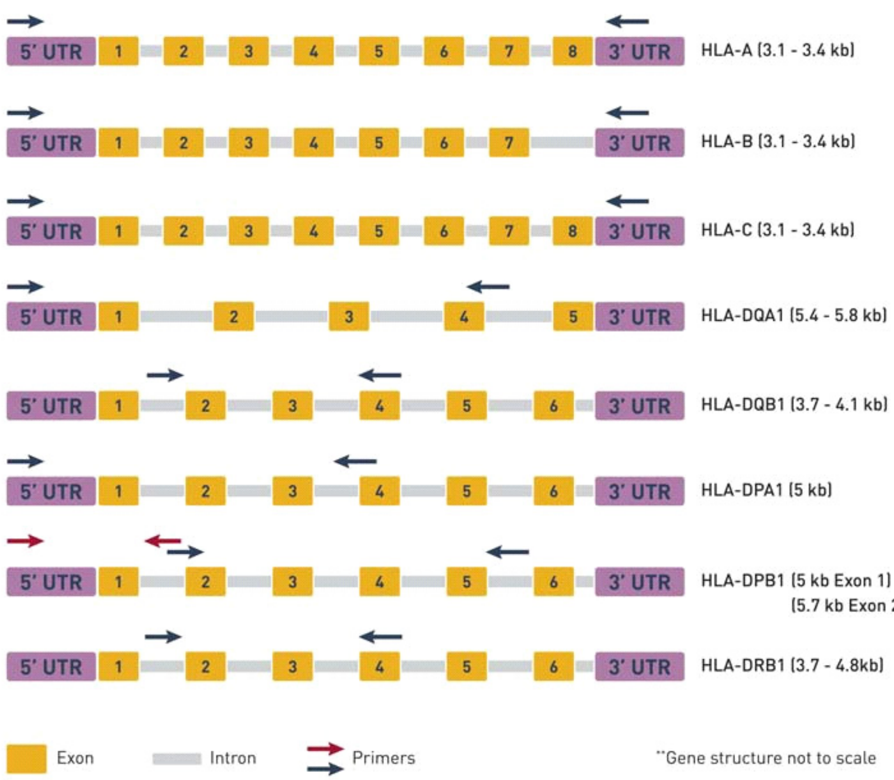

Figure 10 Pooling of Class I and II genes for an individual. Commercial kits such as GenDx NGS-go reagents or a customer-provided assay may be used. Depending on coverage requirements, a pool of Class I and Class II genes can be run on a single SMRT Cell.

Recommendations for Multiplexing NGS-go Amplicons:

Using the NGS-go assay, the following table may be used as a guideline for per-patient multiplexing. The table below includes estimated recommendations for the number of SMRT Cells required to provide sufficient coverage per locus.

Patient Multiplex	HLA Loci	Amplicon Size	Targeted Region	Number of SMRT Cells
24	HLA Class I (A/B/C)	~3.3 kb	Full Length	1
48	HLA Class I (A/B/C)	~3.3 kb	Full Length	1
96	HLA Class I (A/B/C)	~3.3 kb - 5.8 kb	Full Length	2
24	HLA Class I &II (A/B/C/DR/DQ)	~3.3 kb - 5.8 kb	Class I: Full length Class II: Partial	1
48	HLA Class I &II (A/B/C/DR/DQ)	~3.3 kb - 5.8 kb	Class I: Full length Class II: Partial	2
96	HLA Class I &II (A/B/C/DR/DQ)	~3.3 kb - 5.8 kb	Class I: Full length Class II: Partial	4

An example of a high-throughput HLA multiplexing is shown in Figure 11.

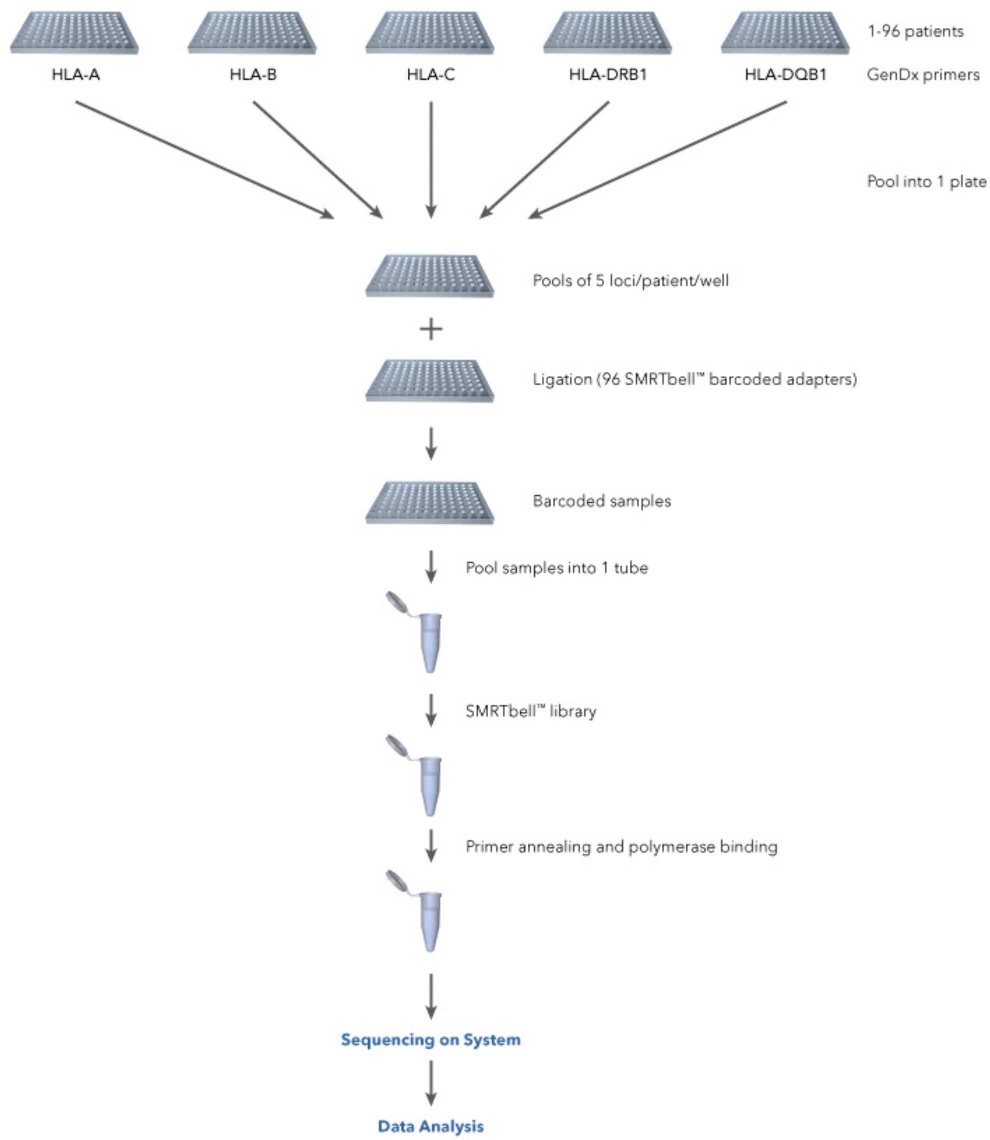

Figure 11 Per-patient Pooling strategy. In this example, GenDx® primer sets for HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 are amplified from 96 patients. The five plates (1 locus x 96 patients) are pooled into one 96-well plate. The 96 samples are then barcoded by ligation with 96 barcoded adapters . The 96 uniquely barcoded samples are then pooled into a single tube which undergoes library construction to generate a SMRTbell library. The number of SMRT Cells required depends on coverage requirements for each amplicon. Pooling allows reduction in workflow and overall project cost.

Qualitative and Quantitative Assessment of PCR Products Prior to Multiplexing and Sequencing

Clean, target-specific PCR products are extremely important for obtaining high-quality sequence information. It is important to perform both quantification and quality checks (for contaminants or impurities) of PCR amplicons before constructing SMRTbell libraries for sequencing. Presence of short non-specific products will result in noisy data.

Some steps that can be taken to ensure high-quality PCR amplicons include:

- Generate high-quality amplicons by ensuring amplification parameters are optimal.
- Use high-quality genomic DNA for PCR.
- Verify amplicon sizes by running on a Bioanalyzer® system (using the DNA 12000 assay kit), Agilent® 2200 TapeStation, agarose gel electrophoresis, or Flash Gels with appropriate molecular weight markers.
- Primer dimers and non-specific PCR products inflate readings using Qubit® or Nanodrop® platforms. They must first be removed by purification, figures 12 and 13.

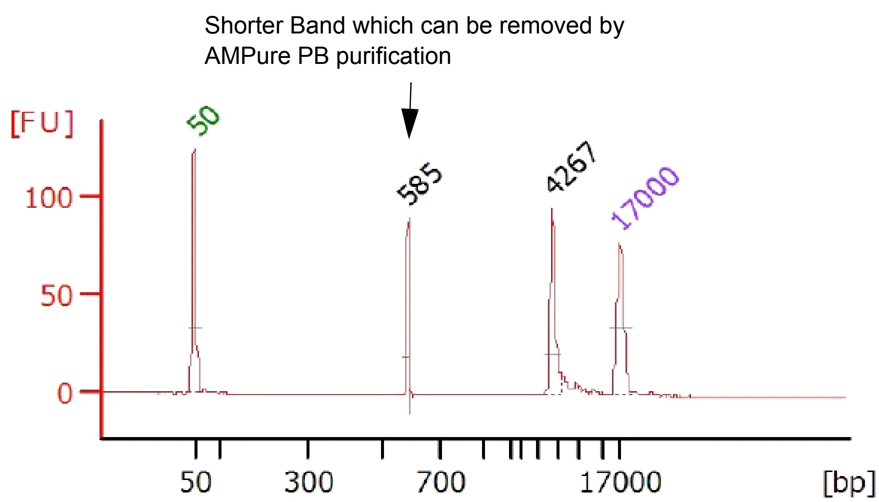

Figure 12 Example of a 4 kb HLA PCR product with contaminating non-specific PCR product (585 bp).

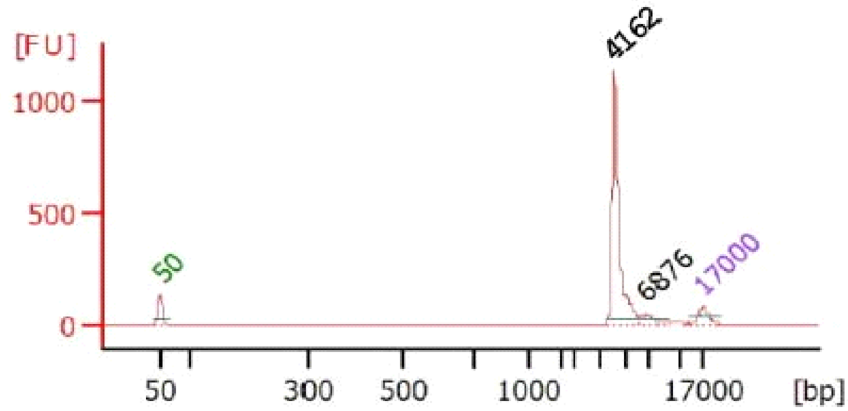

Figure 13: Same sample as Figure 12 purified using AMPure PB beads is shown to remove the 585 bp product.

Remove contaminants and/or non-specific products less than 1 kb by performing AMPure purification. Depending on the size of the unwanted products, use the following AMPure guidelines for purification.

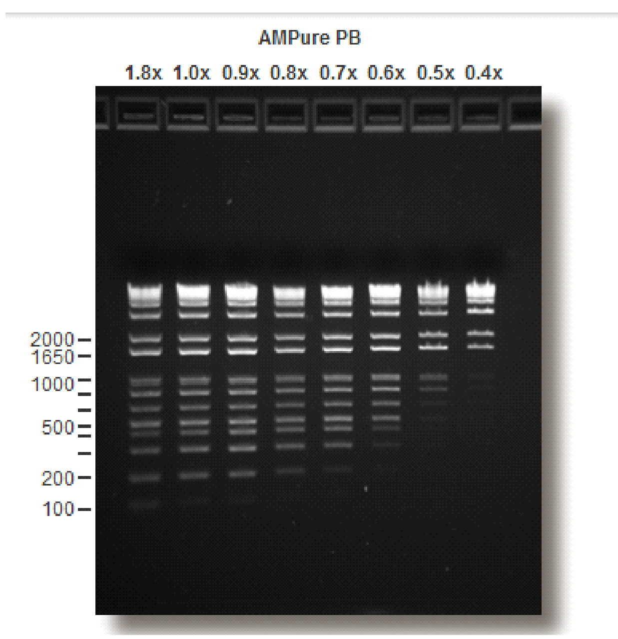

Figure 14 Non-specific PCR products smaller than 1 kb can be removed using AMPure purifications. Use this guideline for determining the required AMPure concentration.

Pooling Calculator:

The Pooling Calculator (www.pacb.com/wp-content/uploads/HLA-Pooling-Calculator.xlsx) is an Excel-based worksheet to assist in pooling experiments designed for samples barcoded with barcoded universal primer or barcoded primer. It does not work well for samples barcoded with barcoded adapter because the library construction workflow and required volumes are different.

The user enters the sample information (e.g. concentration, amplicon size, and volume), and it generates the volume of each sample to be added in the pool. This represents equal-molar pooling.

Sample ID	Sample 1						
Pooling Conditions							
Target Volume of final pool (uL):	20	2	Final Volume of Pool	24.82		Total ng in pool	1825.2
Target concencentration per amplicon (nM) for equimolar pool *minimum concentration required is 2 nM*	4	3	Minimum volume required for pipetting	1.22	If value too low, consider increasing concentration target for equimolar pool	Total ug in library	1.8252
			Number of Amplicons with not enough sample	0			

Calculator outputs total amount available in the library

Calculator displays the minimum targets for pooling based on available library

Amplicon Name	Amplicon size (bp)	Concentration (ng/uL)	Volume available (uL)	Nano molarity (nM)	Available sample (ng)	Minimum targets for Pooling (uL)	Flags	Final concentration of sample in pool
HLA-A	3500	128	25	56.26	1406.59	1.42		3.223732796
HLA-B	3300	138	25	64.34	1608.39	1.24		3.223732796
HLA-C	3300	141	25	65.73	1643.36	1.22		3.223732796
HLA-DRB1	4000	50	25	19.23	480.77	4.16		3.223732796
HLA-DPB1	12000	50	25	6.41	160.26	12.48		3.223732796
HLA-DQB1	9000	109	25	18.63	465.81	4.29		3.223732796

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How to use the calculator:

1. Enter the sample information (amplicon size, concentration, volume) in the blue cells
2. Enter the target volume of final pool in µl. It is recommended to enter the final volume of the sample that goes into DNA Damage repair. Typical volume is 37 µl. If the volume is greater than 37 µl, it is recommended to perform 1x AMPure purification and elute in 37 µl. This is the required volume of the pooled sample that goes into library construction.
3. The value to enter here depends on the required total mass for library construction for a specific amplicon size (see table 1). For example, for amplicons 3 to 10 kb, the amount required is 1 µg.

Modify the volume in the calculator until the value in the “Total µg in library” meets the input requirement.

Library Size	Total Input DNA Required per Pool
1 kb - 3 kb	250 ng - 1 µg
3 kb - 10 kb	1 µg - 5 µg

Table 2 Required sample input for library construction. This is the total mass of the pooled sample.

4. The volume of each sample to be pooled will be displayed in the “Minimum targets for Pooling” column. The sum of the column is the suggested sample mass required for SMRTbell library construction.

Available Procedures:

The procedure to use depends on your workflow requirements.

1. Barcoded Primers
2. Barcoded Universal Primers
3. Barcoded Adapters

Barcoded Primers Procedure

Required Kits and Consumables:

- Template Prep Kit
- DNA/Polymerase Binding Kit
- MagBead Kit
- DNA Sequencing Kit
- SMRT Cells for standard sequencing
- Barcoded adapters

This procedure describes constructing a SMRTbell library from a pool of amplicons. The required total mass of the pooled sample is shown in Table 1.

Pool samples (by equal-mass or equal-volume or equal-molar) and concentrate using AMPure purification (see table below). The recommended elution volume is 37 µl, which is the volume carried into DNA Damage repair.

Library Size	AMPure PB Bead Volumes to Use
250 bp	1.8X
500 bp	1.0X
1 kb to 3 kb	0.6X
3 kb to 10 kb	0.45X

Purify DNA

Perform the following steps (at room temperature) to concentrate your DNA sample. Note that you must use low-adhesion (LoBind) microcentrifuge tubes during the entire template preparation process.

1. Add the appropriate volume (based on insert size) of AMPure PB magnetic beads to the pooled PCR product.

   Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous. Precise volumes are critical to the purification process.

   Consistent and efficient recovery of your sample is critical to successful SMRTbell library preparation. If using this protocol for the first time, we strongly recommend that you process a control sample first. Typical yields from pre-purified DNA (where smaller fragments, primer dimers, or other PCR-related contaminants are already eliminated) are between 80-100%.
2. Mix the bead/amplicon solution thoroughly tapping the tube.
3. Quickly spin down the tube to collect the beads.
4. Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to achieving good library yields. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.
5. Quickly spin down the tube to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. With the tube still on the magnetic bead rack, slowly pipette off the cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add an equal (1:1) volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Wash the beads with freshly prepared 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.

a. Do not remove the tube from the magnetic bead rack.

b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for a 1.5 mL tube or 2 mL for a 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.

c. Do not disturb the bead pellet.

d. After 30 seconds, pipette and discard the 70% ethanol.

9. Repeat step 8 above.

10. Remove residual 70% ethanol and dry the bead pellet.

    a. Remove the tube from the magnetic bead rack and briefly spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.

    b. Place the tube back on magnetic bead rack.

    c. Pipette off any remaining 70% ethanol.

11. Check for any remaining droplets in the tube. If droplets are present, repeat step 10.

12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.

13. Elute the DNA off the beads in **37 µL** of Elution Buffer.

    a. Mix until homogeneous.

    b. Vortex for 1 minute at 2000 rpm.

    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.

    d. Carefully collect the eluted sample.

14. Measure concentration using Qubit system or picogreen quantitation assay.

15. The eluted DNA can be stored overnight at 4ºC or at -20ºC for longer duration.

Repair DNA Damage

Use the following table to repair any DNA damage.

1. Thaw the kit components on ice.
2. In a LoBind microcentrifuge tube, add the following reagents.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
DNA Amplicon Pool	-	-	__µL for 1.0 to 5.0 µg	-
DNA Damage Repair Buffer		10X	5 µL	1X
NAD+		100 X	0.5 µL	1X
ATP Hi		10 mM	5.0 µL	1 mM
dNTP		10 mM	0.5 µL	0.1 mM
DNA Damage Repair Mix			2.0 µL	
H_2O		-	__ µL to adjust to 50* µL	-
Total Volume			50.0 µL	-

*To determine the correct amount of H2O to add, use the actual DNA amount from row 1 of above table.

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin down tube contents with a quick spin in a microfuge.
5. Incubate at 37ºC for 20 minutes to 1 hour, then return reaction to 4ºC for 1 minute.

Repair Ends

Use the following table to prepare your reaction and then purify the DNA.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
DNA (Damage Repaired) Amplicons	-		50 µL	-
End Repair Mix		20 X	2.5 µL	1X
Total Volume			52.5 µL	-

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down the contents of the tube with a quick spin in a microfuge.
3. Incubate at 25ºC for 5 minutes, then return the reaction to 4ºC. Proceed to the next step.

Purify the DNA

Perform the following steps at room temperature. Note that you must use low-adhesion (LoBind) microcentrifuge tubes during the entire template preparation process.

1. Add the appropriate volume of AMPure PB magnetic beads to the End-Repair reaction.

   Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.
2. Mix the bead/DNA solution thoroughly by tapping the tube.
3. Quickly spin down the tube to collect the beads.
4. Transfer the tube to a VWR vortex mixer and allow the DNA to bind to beads by shaking at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

5. Spin down the tube to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. Slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Once the supernatant has been removed, add 70% ethanol all the way to the rim of the Eppendorf tube (if 1.5 mL, then add ~1.5 mL). Wash beads with the 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.
9. Once the tube has been filled, start removing the ethanol and discard it. Then repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove the tube from the magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

    Turn the tube to verify that the ethanol has been fully removed. If the bead pellet is dry, it will stay on the wall in the same spot despite not being on the magnet bead rack.

11. If any remaining droplets of ethanol are present in the tube, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds. (Do **not** exceed 2 minutes or the beads can overdry.)
13. Remove the tube from the magnetic rack and add **30 µL** of Elution Buffer to the sample. Elute the DNA off the beads by vortexing for 10 minutes at 2000 rpm.
    a. Quickly spin (or pulse) down the tube to collect the beads.
    b. Put the tube back on the magnetic rack to elute the sample from the beads.
    c. Transfer the eluted sample to a fresh tube.
    d. The bead tube can be discarded.

Stopping Point

14. The End-Repaired DNA can be stored overnight at 4ºC or at -20ºC for longer duration.

Blunt-End Ligation of SMRTbell™ Adapters to the End-Repaired Amplicons

During this step, blunt hairpins are ligated to repaired fragment ends.

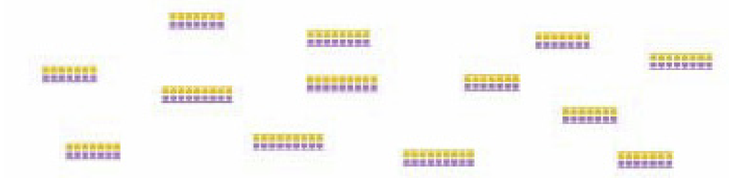


Figure 1 Repaired Amplicon Ends

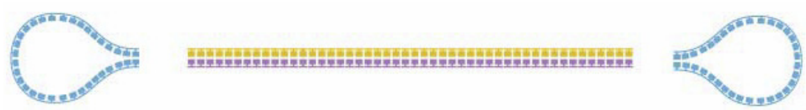


Figure 2 Blunt Hairpin Adapters and Insert DNA Amplicon Ready for Ligation

To ligate the hairpins (SMRTbell adapters) to the DNA fragments, you will need BLUNT hairpin adapters. These are shipped as 20 µM oligo-nucleotide stock and are pre-annealed. This reaction can be scaled for the number of library samples being prepared.

Blunt-End Ligation Reaction

In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown. Note that you may need to add water to achieve the desired DNA volume. **If preparing a Master Mix, ensure that the adapter is NOT mixed with the ligase prior to introduction of the inserts**. Add the adapter to the well with the DNA. All other components should be added to the Master Mix.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
DNA (End Repaired)	-		29 to 30 µL	
Blunt Adapter (20 µM)		20 µM	1.0 µL	0.5 µM
Mix before proceeding				
Template Prep Buffer		10X	4.0 µL	1X
ATP Lo		1 mM	2.0 µL	0.05 mM
Mix before proceeding				
Ligase[a] (Do Not Add to Master Mix)		30 U/µL	1.0 µL	0.75 U/µL
H_2O			to 40.0 µL	
Total Volume	-	-	40 µL	-

a. The Ligase Enzyme tube should remain closed and on ice when not frozen.

If the insert size or input amount deviates from this table, calculate the amount of annealed blunt adapter to be added to the reaction using the following equation. Be sure to keep a minimum of 32.5 fold excess of hairpin adapters. If the input DNA is less than 500 ng, increase the hairpin adapter concentration 100X to 200X to minimize chimera formation and maximize library yield. When increasing the SMRTbell adapter concentration in this reaction, be sure to perform triple AMPure PB bead purifications at the end of the library preparation process to clean up or reduce adapter dimer contamination which may result in a drop in sequencing performance.

Total µg of DNA insert X * 10^6 * 1/650 X 1/insert size in bp = ***X*** picomoles of DNA available for ligation

X picomoles of DNA available for ligation X 100 = Total excess annealed adapters (***Y***)

Y/20 (20 µM annealed adaptor stock) = ***Z*** total µL of annealed adaptor to be added to the reaction

If scaling of the reaction volume is necessary, keep the buffer and enzyme concentrations proportional to the recommended amounts shown above.

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down the tube contents with a quick spin in a microfuge.
3. Incubate at 25ºC for 15 minutes (for input material greater than 1 µg). Overnight incubation at 25ºC is recommended for input material less than 1 µg. At this point, the ligation can be extended up to 24 hours to maximize ligation efficiency, or cooled to 4ºC for storage of up to 24 hours.
4. Incubate at 65ºC for 10 minutes to inactivate the ligase (if ligase is not inactivated at this step it will stay bound to DNA and the Exo step will not remove incompletely ligated products), then return the reaction to 4ºC. **You must proceed with adding exonuclease after this step.**

No Stopping

Add Exonuclease and Incubate

Add exonuclease to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.	Volume
Ligated DNA			40 µL
ExoIII		100 U/µL	1.0 µL
ExoVII		10 U/µL	1.0 µL
Total Volume	-	-	42 µL

5. Mix the reaction well by pipetting or flicking the tube.
6. Spin down the tube contents with a quick spin in a microfuge.
7. Incubate at 37º C for 1 hour, then return the reaction to 4ºC. Do not exceed the 1 hour incubation time. **You must proceed with AMPure PB bead purification after this step.**

Purify SMRTbell™ Templates

In this purification process, there are two (2) distinct and consecutive AMPure PB bead purification steps. Perform all purification steps at room temperature to adequately remove enzymes (exonucleases, ligases, etc.) and ligation products smaller than 0.4 kb, such as adapter dimers.

The AMPure PB bead concentration for both steps depends on the library size. Use the recommended concentrations shown below:

AMPure PB Bead Purification Step #1:

1. Add the appropriate volume of AMPure PB magnetic beads to the exonuclease-treated ligation reaction. See the table below:

Insert Size	AMPure PB Bead Volumes to Use
250 bp	1.8X
500 bp	1.0X
1 kb to 3 kb	0.6X
3 kb to 10 kb	0.45X

2. Mix the bead/DNA solution thoroughly by tapping the tube.
3. Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.
4. Transfer the tube to a VWR vortex mixer and allow the DNA to bind to beads by shaking at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

5. Spin down the tube (for 1 second) to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. Slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Once the supernatant has been removed, add 70% ethanol all the way to the rim of the Eppendorf tube (if 1.5 mL, then add ~1.5 mL). Wash beads with the 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.
9. Once the tube has been filled, start removing the ethanol and discard it. Then repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove the tube from the magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

11. If any remaining droplets of ethanol are present in the tube, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds. (Do **not** exceed 2 minutes or the beads can over dry.)
13. Remove the tube from the magnetic rack and add **50 µL** of Elution Buffer to the sample. Elute the DNA off the beads by vortexing for 10 minutes at 2000 rpm.
    a. Mix until homogeneous.
    b. Vortex for 1 minute at 2000 rpm.
    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
    d. Carefully collect the eluted sample.
    e. The bead tube can be discarded.

AMPure PB and Purification Step #2:

1. Add the appropriate volume of AMPure PB magnetic beads to the **50 µL** of eluted DNA from the first AMPure PB bead purification step above.
2. Mix the bead/DNA solution thoroughly by tapping the tube.
3. Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.
4. Transfer the tube to a VWR vortex mixer and allow the DNA to bind to beads by shaking at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

5. Spin down the tube (for 1 second) to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. Slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Once the supernatant has been removed, add 70% ethanol all the way to the rim of the Eppendorf tube (if 1.5 mL, then add ~1.5 mL). Wash beads with the 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.
9. Once the tube has been filled, start removing the ethanol and discard it. Then repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove the tube from the magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

11. If any remaining droplets of ethanol are present in the tube, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds. (Do **not** exceed 2 minutes or the beads can over dry.)
13. Remove the tube from the magnetic rack and add **8 µL to 10 µL** of Elution Buffer to the sample. Elute the DNA off the beads by vortexing for 10 minutes at 2000 rpm.
    a. Mix until homogeneous.
    b. Vortex for 1 minute at 2000 rpm.
    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
    d. Carefully collect the eluted sample.
    e. The bead tube can be discarded.
14. Verify your DNA amount and concentration on a Bioanalyzer instrument using a High Sensitivity DNA Analysis Kit, or check quantitation using Qubit dsDNA HS Assay Kit. If there is too little sample, estimate the concentration based on 10% yield of input amount into the damage repair step.

Barcoded Universal Primers Procedure

This procedure describes methods for generating barcoded PCR products using PacBio Barcoded Universal Primers (BUP).

The procedure provides recommendations for amplifying targets using primers tailed with two universal sequences. The amplified products are further amplified using barcoded universal primers to incorporate up to 96 PacBio 16-bp barcodes. The barcoded PCR products are pooled for SMRTbell library construction and subsequently sequenced in the PacBio System.

Required Kits and Consumables

- PacBio® Template Prep Kit
- DNA/Polymerase Binding Kit
- MagBead Kit
- DNA Sequencing Kit
- Control Complex
- AMPure® PB Beads
- SMRT® Cells
- PCR amplification assays (home brew or GenDx NGS-go® kit)
- PCR Enzyme Phusion® (New England BioLabs) or preferred enzyme
- Low-adhesion (LoBind) microcentrifuge tubes
- PacBio Barcoded Universal F/R Primers Plate - 96

Multiplexing using Barcoded Universal Primers:

When using this procedure, target-specific primers must be tailed with universal sequences, followed by a second amplification using the PacBio Barcoded Universal F/R Primers.

Designing Target-Specific Primers Tailed with Universal Sequences

It is highly recommended to meet the following requirements when designing primers.

1. Add a 5’ block (e.g., 5’ NH4-C6) to ensure that carry over amplicons, from first round PCR, are not ligated to the SMRTbell adapters in subsequent steps.
2. HPLC purified.
3. Use the following primer format:

Primer Type	Universal Sequence	Template Specific Sequence	Primer to Order
Forward_Internal_ PCR_Primer	/5AmMC6/ gcagtcgaacatgtagctgactcaggtcac	FOR EXAMPLE1	/5AmMC6/ gcagtcgaacatgtagctgactc aggtcacFOR EXAMPLE1
Reverse_Internal_ PCR_Primer	/5AmMC6/ tggatcacttgtgcaagcatcacatcgtag	FOR EXAMPLE2	/5AmMC6/ tggatcacttgtgcaagcatcaca tcgtagFOR EXAMPLE2

First Round of PCR: Recommendations for Target-Specific Primers Tailed with Universal Sequence

The procedure below was optimized using high-fidelity Phusion Hot Start II for PCR. Other high-fidelity polymerases may be used. We recommend optimizations before proceeding.

1. Prepare the following reaction per sample:

Component	Stock Conc.	Final Conc.	1X
HPLC Water			13.75
PHusion Buffer HF	5X	1X	5
dNTP	2 mM	0.2 mM	2.5
DNA	1 ng/µLa		1
Universal sequence tagged Primer 1b and Primer 2[b]	2 µM each	0.2 µM	2.5
Phusion HF	2 U/µL		0.25
Total Volume	-	-	25

a. General guideline: Optimization is recommended to determine the appropriate amount of template.
b. Five prime end must be blocked (e.g., 5’ NH4-C6)

2. Mix gently by tapping the tube. Quick spin the tube.

3. Perform amplification using the following cycling parameters. Amplification depends on the sample and the target-specific primers. Optimization is highly recommended.

Step	Temperature	Time
1	98ºC	30 seconds[a]
2	98ºC[b]	15 seconds
3	N[c]	15 seconds
4	72ºC	X min[d]
5	Repeat steps 2 to 4 (20 cycles total)e	-
6	72ºC	7 minutes
7	4ºC	Hold

a. Initial denaturation is dependent on template complexity (30 seconds – 3 minutes).
b. Temperature is dependent on the polymerase.
c. N in step 3 is defined by the user depending on Primers 1 and 2.
d. X in step 4 is defined by the user depending on the insert size.
e. The number of cycles in step 5 can be increased to 20-25 cycles for visual inspection on an agarose gel.

4. After amplification, perform visual inspection of the PCR products on an agarose gel.
5. Proceed to the second round of PCR.

Second Round PCR: Recommendations for Target-Specific Primers Tailed with Universal Sequence

The procedure below was optimized using high-fidelity Phusion Hot Start II for PCR. Other high-fidelity polymerases may be used. We recommend to perform optimizations before proceeding.

1. Prepare the following reaction per sample:

Component	Stock Conc.	Final Conc.	1X
HPLC Water			13.75
PHusion Buffer HF	5X	1X	5
dNTP	2 mM	0.2 mM	2.5
Round 1 PCR	1 ng/µL[a]		1
BUP Primer s[b]	2 µM each	0.2 µM each	2.5
Phusion HF	2 U/µL	0.02 U/µL	0.25
Total Volume	-	-	25

a. General guideline: Optimization is recommended to determine the appropriate amount of round 1 PCR product.
b. Barcoded Universal Forward and Reverse primers from the 96-well barcode plate.

2. Mix gently by tapping the tube. Quick spin the tube.
3. Perform amplification using the following cycling parameters. Amplification depends on the sample and the target-specific primers. Optimization is highly recommended.

Step	Temperature	Time
1	98ºC	30 seconds
2	98ºC	15 seconds
3	64ºC[a]	15 seconds
4	72ºC	X min[b]
5	Repeat steps 2 to 4 (20 cycles total)[c]	-
6	72ºC	7 minutes
7	4ºC	Hold

a. Recommended for Barcoded Universal Primers.
b. Defined by the user depending on the insert size.
c. The number of cycles can be increased to 20-25 cycles for visual inspection on an agarose gel.

4. After amplification, purify samples using AMPure beads and measure concentration using a Qubit system or picogreen quantitation assay. Measure amplicon size using a BioAnalyzer instrument. For high-throughput applications, perform visual inspection of the products on an agarose gel.
5. Pool samples by equal-volume, equal-mass or equal-molarity (See Pooling Calculator Section on pages 14-15 for guidance on pooling).
6. Concentrate the pooled sample and proceed to the next section.

Purify DNA

Perform the following steps (at room temperature) to concentrate your DNA sample. Note that you must use low-adhesion (LoBind) microcentrifuge tubes during the entire template preparation process.

1. Add the appropriate volume (based on insert size) of AMPure PB magnetic beads to the pooled PCR product.

   Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous. Precise volumes are critical to the purification process.

   Consistent and efficient recovery of your sample is critical to successful SMRTbell library preparation. If using this protocol for the first time, we strongly recommend that you process a control sample first. Typical yields from pre-purified DNA (where smaller fragments, primer dimers, or other PCR-related contaminants are already eliminated) are between 80-100%.
2. Mix the bead/amplicon solution thoroughly tapping the tube.
3. Quickly spin down the tube to collect the beads.
4. Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to achieving good library yields. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.
5. Quickly spin down the tube to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.

7. With the tube still on the magnetic bead rack, slowly pipette off the cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add an equal (1:1) volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.

8. Wash the beads with freshly prepared 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.

   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for a 1.5 mL tube or 2 mL for a 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.

9. Repeat step 8 above.

10. Remove residual 70% ethanol and dry the bead pellet.

    a. Remove the tube from the magnetic bead rack and briefly spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

11. Check for any remaining droplets in the tube. If droplets are present, repeat step 10.

12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.

13. Elute the DNA off the beads in **37 µL** of Elution Buffer.

    a. Mix until homogeneous.
    b. Vortex for 1 minute at 2000 rpm.
    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
    d. Carefully collect the eluted sample.

14. Measure concentration using Qubit system or picogreen quantitation assay.

15. The eluted DNA can be stored overnight at 4ºC or at -20ºC for longer duration.

Repair DNA Damage

Use the following table to repair any DNA damage.

1. Thaw the kit components on ice.
2. In a LoBind microcentrifuge tube, add the following reagents.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
DNA Amplicon Pool	-	-	__µL for 1.0 to 5.0 µg	-
DNA Damage Repair Buffer		10X	5.0 µL	1X
NAD+		100 X	0.5 µL	1X
ATP Hi		10 mM	5.0 µL	1mM
dNTP		10 mM	0.5 µL	0.1 mM
DNA Damage Repair Mix			2.0 µL	
H_2O		-	__ µL to adjust to 50* µL	-
Total Volume			50.0 µL	-

*To determine the correct amount of H2O to add, use the actual DNA amount from row 1 of above table.

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin down tube contents with a quick spin in a microfuge.
5. Incubate at 37ºC for 20 minutes or longer, then return reaction to 4ºC for 1 minute. This is a safe stopping point.

Repair Ends

Use the following table to prepare your reaction and then purify the DNA.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
DNA (Damage Repaired)	-		50 μL	-
End Repair Mix		20 X	2.5 μL	1X
Total Volume			52.5 μL	-

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down the contents of the tube with a quick spin in a microfuge.
3. Incubate at 25ºC for 5 minutes, then return the reaction to 4ºC. Proceed to the next step.

Purify DNA

Perform the following steps (at room temperature) to concentrate your DNA sample. Note that you must use low-adhesion (LoBind) microcentrifuge tubes during the entire template preparation process.

1. Add the appropriate volume (based on insert size) of AMPure PB magnetic beads to the pooled PCR product.

   Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous. Precise volumes are critical to the purification process.

   Consistent and efficient recovery of your sample is critical to successful SMRTbell library preparation. If using this protocol for the first time, we strongly recommend that you process a control sample first. Typical yields from pre-purified DNA (where smaller fragments, primer dimers, or other PCR-related contaminants are already eliminated) are between 80-100%.

2. Mix the bead/amplicon solution thoroughly tapping the tube.
3. Quickly spin down the tube to collect the beads.
4. Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to achieving good library yields. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.
5. Quickly spin down the tube to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. With the tube still on the magnetic bead rack, slowly pipette off the cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add an equal (1:1) volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Wash the beads with freshly prepared 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for a 1.5 mL tube or 2 mL for a 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.
9. Repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove the tube from the magnetic bead rack and briefly spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

11. Check for any remaining droplets in the tube. If droplets are present, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.
13. Elute the DNA off the beads in **30 µL** of Elution Buffer.
    a. Mix until homogeneous.
    b. Vortex for 1 minute at 2000 rpm.
    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
    d. Carefully collect the eluted sample.
14. The eluted DNA can be stored overnight at 4ºC or at -20ºC for longer duration.

Blunt-Ligation Reaction

In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown. Note that you may need to add water to achieve the desired DNA volume. **If preparing a Master Mix, ensure that the adapter is NOT mixed with the ligase prior to introduction of the inserts**. Add the adapter to the well with the DNA. All other components should be added to the Master Mix.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
DNA (End Repaired)	-		29 to 30 µL	
Blunt Adapter (20 µM)		20 µM	1.0 µL	0.5 µM
Mix before proceeding				
Template Prep Buffer		10X	4.0 µL	1X
ATP Lo		1 mM	2.0 µL	0.05 mM
Mix before proceeding				
Ligase[a] (Do Not Add to Master Mix)		30 U/µL	1.0 µL	0.75 U/µL
H_2O			to 40.0 µL	
Total Volume	-	-	40 µL	-

a. The Ligase Enzyme tube should remain closed and on ice when not frozen.

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down the tube contents with a quick spin in a microfuge.
3. Incubate at 25ºC for 15 minutes. At this point, the ligation can be extended up to 24 hours to maximize ligation efficiency, or cooled to 4ºC for storage of up to 24 hours.
4. Incubate at 65ºC for 10 minutes to inactivate the ligase, then return the reaction to 4ºC. **You must proceed with adding exonuclease after this step.**

No Stopping

Add Exonuclease and Incubate

Add exonuclease to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.	Volume
Ligated DNA			40 µL
ExoIII		100 U/µL	1.0 µL
ExoVII		10 U/µL	1.0 µL
Total Volume	-	-	42 µL

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down the tube contents with a quick spin in a microfuge.
3. Incubate at 37º C for 1 hour, then return the reaction to 4ºC. Do not exceed the 1 hour incubation time. **You must proceed with AMPure PB bead purification after this step.**

Purify SMRTbell™ Templates

In this purification process, there are two (2) distinct and consecutive AMPure PB bead purification steps. The AMPure PB bead concentration for both steps depends on the library size. Use the recommended concentrations shown.

Insert Size	AMPure PB Bead Volumes to Use
250 bp	1.8X
500 bp	1.0X
1 kb to 3 kb	0.6X
3 kb to 10 kb	0.45X

AMPure PB Bead Purification Step #1:

1. Add the appropriate volume of AMPure PB magnetic beads to the exonuclease-treated ligation reaction. See the table below:
2. Mix the bead/DNA solution thoroughly by tapping the tube.
3. Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.
4. Transfer the tube to a VWR vortex mixer and allow the DNA to bind to beads by shaking at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

5. Spin down the tube (for 1 second) to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. Slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Once the supernatant has been removed, add 70% ethanol all the way to the rim of the Eppendorf tube (if 1.5 mL, then add ~1.5 mL). Wash beads with the 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.
9. Once the tube has been filled, start removing the ethanol and discard it. Then repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove the tube from the magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

11. If any remaining droplets of ethanol are present in the tube, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds. (Do **not** exceed 2 minutes or the beads can over dry.)
13. Remove the tube from the magnetic rack and add **50 µL** of Elution Buffer to the sample. Elute the DNA off the beads by vortexing for 10 minutes at 2000 rpm.
    a. Mix until homogeneous.
    b. Vortex for 1 minute at 2000 rpm.
    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
    d. Carefully collect the eluted sample.
    e. The bead tube can be discarded.

AMPure PB and Purification Step #2:

1. Add the appropriate volume of AMPure PB magnetic beads to the **50 µL** of eluted DNA from the first AMPure PB bead purification step above.
2. Mix the bead/DNA solution thoroughly by tapping the tube.
3. Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.
4. Transfer the tube to a VWR vortex mixer and allow the DNA to bind to beads by shaking at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

5. Spin down the tube (for 1 second) to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. Slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Once the supernatant has been removed, add 70% ethanol all the way to the rim of the Eppendorf tube (if 1.5 mL, then add ~1.5 mL). Wash beads with the 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.
9. Once the tube has been filled, start removing the ethanol and discard it. Then repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove the tube from the magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

11. If any remaining droplets of ethanol are present in the tube, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds. (Do **not** exceed 2 minutes or the beads can over dry.)
13. Remove the tube from the magnetic rack and add **8 µL to 10 µL** of Elution Buffer to the sample. Elute the DNA off the beads by vortexing for 10 minutes at 2000 rpm.
    a. Mix until homogeneous.
    b. Vortex for 1 minute at 2000 rpm.
    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
    d. Carefully collect the eluted sample.
    e. The bead tube can be discarded.
14. Verify your DNA amount and concentration on a Bioanalyzer instrument using a High Sensitivity DNA Analysis Kit, or check quantitation using Qubit dsDNA HS Assay Kit. If there is too little sample, estimate the concentration based on 10% yield of input amount into the damage repair step.

Barcoded Adapter Procedure

This procedure describes methods for generating SMRTbell libraries using barcoded adapters. Multiple genes from a given individual are pooled, followed by ligation to a unique barcoded SMRTbell adapter (Figure 10). Once tagged, barcoded samples are pooled for SMRTbell library construction. Figure 10 is an example of multiplexing multiple genes from multiple individuals.

Required Kits and Consumables:

- Template Prep Kit
- DNA/Polymerase Binding Kit
- MagBead Kit
- DNA Sequencing Kit
- SMRT Cells for standard sequencing
- Barcoded adapters

Pooling and Sample Requirements

As a general guideline, pool by size to reduce loading bias across alleles. See “Designing HLA Pools” section for pooling Class I and Class II genes. Best results are observed from equal-molar pooling.

Note: The procedure requires a volume 4 µl of the pooled sample into a single-tube end repair and ligation reaction. If the volume exceeds 4 µl, the sample must be concentrated using AMPure PB beads.

One-Step End-Repair and Ligation Reaction

Each individual sample must first go through this step before pooling. This step repairs the ends of the DNA, followed by ligation with the barcoded adapters in a single-tube reaction.

1. Make a pre-mix of the following reaction and store in ice. Account for any pipetting errors when making a pre-mix.

Reagent	Tube Cap Color	Stock Conc.	For 1X	For ‘__’ Samples + Overage
Template Prep Buffer		10X	1.0 µL	µL
ATP Hi		10 mM	1.0 µL	µL
dNTP		10 mM	0.1 µL	µL
End Repair Mix		20X	0.5 µL	µL
Ligase		30 U/µL	0.3 µL	µL
H_2O		-	0.6 µL	µL
Total Volume			3.5 µL	µL

2. Mix by tapping the tube. Do a quick spin down of the tube. Note that the pre-mix must be used immediately.
3. Transfer 3.5 µL aliquots of the above pre-mix to a 96-well plate or LoBind Microcentrifuge tubes. Set aside in ice until ready to use.
4. Prepare the samples by diluting each sample to a volume of 4 µL. Concentrate sample with AMPure PB if volume is greater than 4 µL
5. In a separate plate or tubes, add the template and barcoded adapter in the order listed (mix after each addition). Keep reagents and samples on ice at all times.

Component	Volume	Final Concentration
Sample	4.0 µL	
Barcoded Adapter	2.5 µL	5.0 µM
Total	6.5 µL	

6. Finally, add 3.5 µL of the premix to 6.5 µL of the diluted/barcoded adapter mix for a total of 10 µL per reaction. If working with less than 96 barcodes, the foil seal of the barcoded adapter plate can be easily pierced with a pipet tip. Any untouched adapter barcodes can be stored at -20ºC for future use. Each barcode in the well is for one time use only. See the *User Bulletin - Barcode Plate Mapping* for additional information.
7. Incubate as follows:

Step	Temperature	Time
1	37ºC	20 minutes
2	25ºC	15 minutes
3	65ºC	15 minutes
4	4ºC	Hold

8. After the End-Repair and Ligation reaction, pool the 10 µL volume reactions.
9. Measure the volume and perform AMPure PB bead purification. Use the table below to determine the appropriate concentration of AMPure PB beads to use:

Insert Size	AMPure PB Bead Volumes to Use
250 bp	1.8X
500 bp	1.0X
1 kb to 3 kb	0.6X
3 kb to 10 kb	0.45X

10. Measure the concentration using a Qubit system.

Purify DNA

Perform the following steps (at room temperature) to concentrate your DNA sample. Note that you must use low-adhesion (LoBind) microcentrifuge tubes during the entire template preparation process.

1. Add the appropriate volume (based on insert size) of AMPure PB magnetic beads to the pooled PCR product.

   Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous. Precise volumes are critical to the purification process.

   Consistent and efficient recovery of your sample is critical to successful SMRTbell library preparation. If using this protocol for the first time, we strongly recommend that you process a control sample first. Typical yields from pre-purified DNA (where smaller fragments, primer dimers, or other PCR-related contaminants are already eliminated) are between 80-100%.
2. Mix the bead/amplicon solution thoroughly tapping the tube.
3. Quickly spin down the tube to collect the beads.
4. Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to achieving good library yields. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.
5. Quickly spin down the tube to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. With the tube still on the magnetic bead rack, slowly pipette off the cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add an equal (1:1) volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Wash the beads with freshly prepared 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.

a. Do not remove the tube from the magnetic bead rack.

b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for a 1.5 mL tube or 2 mL for a 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.

c. Do not disturb the bead pellet.

d. After 30 seconds, pipette and discard the 70% ethanol.

9. Repeat step 8 above.

10. Remove residual 70% ethanol and dry the bead pellet.

    a. Remove the tube from the magnetic bead rack and briefly spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.

    b. Place the tube back on magnetic bead rack.

    c. Pipette off any remaining 70% ethanol.

11. Check for any remaining droplets in the tube. If droplets are present, repeat step 10.

12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.

13. Elute the DNA off the beads in **37 µL** of Elution Buffer.

    a. Mix until homogeneous.

    b. Vortex for 1 minute at 2000 rpm.

    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.

    d. Carefully collect the eluted sample.

14. Measure concentration using Qubit system.

15. The eluted DNA can be stored overnight at 4ºC or at -20ºC for longer duration.

Repair DNA Damage

DNA Damage Repair is recommended for all libraries. Use the following table to prepare the reaction, followed by exonuclease digestion.

Library Size	Total Input DNA Required per Pool
1 kb - 3 kb	250 ng - 1 µg
3 kb - 10 kb	1 µg - 5 1 µg

Use the following table to repair any DNA damage.

1. Thaw the kit components on ice.
2. In a LoBind microcentrifuge tube, add the following reagents.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.
DNA Amplicon Pool	-	-	__µL for 1.0 to 5.0 µg	-
DNA Damage Repair Buffer	●	10X	5 µL	1X
NAD+	●	100 X	0.5 µL	1X
ATP Hi	●	10 mM	5.0 µL	1mM
dNTP	●	10 mM	0.5 µL	0.1 mM
DNA Damage Repair Mix	●		2.0 µL	
H_2O		-	__ µL to adjust to 50* µL	-
Total Volume			50.0 µL	-

*To determine the correct amount of H2O to add, use the actual DNA amount from row 1 of above table.

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin down tube contents with a quick spin in a microfuge.
5. Incubate at 37ºC for 20 minutes, then return reaction to 4ºC. Proceed to the next step.

Add Exonuclease and Incubate

Add exonuclease to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.	Volume
Ligated DNA			50 µL
ExoIII		100 U/µL	1.0 µL
ExoVII		10 U/µL	1.0 µL
Total Volume	-	-	52 µL

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down the tube contents with a quick spin in a microfuge.
3. Incubate at 37º C for 1 hour, then return the reaction to 4ºC. Do not exceed the 1 hour incubation time. **You must proceed with AMPure PB bead purification after this step.**

Purify SMRTbell™ Templates

In this purification process, there are two (2) distinct and consecutive AMPure PB bead purification steps. The AMPure PB bead concentration for both steps depends on the library size. Use the recommended concentrations shown.

AMPure PB Bead Purification Step #1:

1. Add the appropriate volume of AMPure PB magnetic beads to the exonuclease-treated ligation reaction. See the table below:

Insert Size	AMPure PB Bead Volumes to Use
250 bp	1.8X
500 bp	1.0X
1 kb to 3 kb	0.6X
3 kb to 10 kb	0.45X

2. Mix the bead/DNA solution thoroughly by tapping the tube.
3. Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.
4. Transfer the tube to a VWR vortex mixer and allow the DNA to bind to beads by shaking at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

5. Spin down the tube (for 1 second) to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. Slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Once the supernatant has been removed, add 70% ethanol all the way to the rim of the Eppendorf tube (if 1.5 mL, then add ~1.5 mL). Wash beads with the 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.
9. Once the tube has been filled, start removing the ethanol and discard it. Then repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove the tube from the magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

11. If any remaining droplets of ethanol are present in the tube, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds. (Do **not** exceed 2 minutes or the beads can over dry.)
13. Remove the tube from the magnetic rack and add **50 µL** of Elution Buffer to the sample. Elute the DNA off the beads by vortexing for 10 minutes at 2000 rpm.
    a. Mix until homogeneous.
    b. Vortex for 1 minute at 2000 rpm.
    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
    d. Carefully collect the eluted sample.
    e. The bead tube can be discarded.

AMPure PB and Purification Step #2:

1. Add the appropriate volume of AMPure PB magnetic beads to the **50 µL** of eluted DNA from the first AMPure PB bead purification step above.
2. Mix the bead/DNA solution thoroughly by tapping the tube.
3. Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.
4. Transfer the tube to a VWR vortex mixer and allow the DNA to bind to beads by shaking at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous.

   We recommend using a VWR vortex mixer with a foam microtube attachment. If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.

5. Spin down the tube (for 1 second) to collect beads.
6. Place the tube in a magnetic bead rack until the beads collect to the side of the tube. The actual time required to collect the beads to the side depends on the volume of beads added.
7. Slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.

   If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.
8. Once the supernatant has been removed, add 70% ethanol all the way to the rim of the Eppendorf tube (if 1.5 mL, then add ~1.5 mL). Wash beads with the 70% ethanol.

   Note that 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
   a. Do not remove the tube from the magnetic bead rack.
   b. Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds.
   c. Do not disturb the bead pellet.
   d. After 30 seconds, pipette and discard the 70% ethanol.
9. Once the tube has been filled, start removing the ethanol and discard it. Then repeat step 8 above.
10. Remove residual 70% ethanol and dry the bead pellet.
    a. Remove the tube from the magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube.
    b. Place the tube back on magnetic bead rack.
    c. Pipette off any remaining 70% ethanol.

11. If any remaining droplets of ethanol are present in the tube, repeat step 10.
12. Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds. (Do **not** exceed 2 minutes or the beads can over dry.)
13. Remove the tube from the magnetic rack and add **8 µL to 10 µL** of Elution Buffer to the sample. Elute the DNA off the beads by vortexing for 10 minutes at 2000 rpm.
    a. Mix until homogeneous.
    b. Vortex for 1 minute at 2000 rpm.
    c. Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
    d. Carefully collect the eluted sample.
    e. The bead tube can be discarded.
14. Verify your DNA amount and concentration on a Bioanalyzer instrument using a High Sensitivity DNA Analysis Kit, or check quantitation using Qubit dsDNA HS Assay Kit. If there is too little sample, estimate the concentration based on 10% yield of input amount into the damage repair step.

The library is now ready for primer annealing, polymerase binding, and sequencing.

Anneal and Bind SMRTbell™ Templates

You must have the PacBio Binding Kit and use LoBind microcentrifuge tubes for this step. To anneal sequencing primer and bind polymerase to SMRTbell templates, see the Calculator. For more information about using the calculator, see the relevant Pacific Biosciences Guide.

Bind Complex to MagBeads

To maximize yield, use MagBead loading for libraries made using this procedure. The Calculator provides recommended sample concentrations for large scale and custom (small) scale binding; however, we recommend that you titrate samples to determine optimal loading concentrations for specific libraries.

Sequence on the Instrument

For information on how to prepare and sequence using MagBeads, see the document **Pacific Biosciences Procedure & Checklist - Preparing MagBeads for Sequencing**.

How Long Until Data is Ready for Secondary Analysis?

Currently, primary analysis generally takes ~50-60% of the length of the movie time to complete, or ~1 hour for a ~2-hour movie. Allow approximately 50% of the movie time after primary analysis ends before data gets to storage, and is ready for secondary analysis. Data transfer from the podium will take additional time beyond that, and depends almost entirely on the speed of your internal network.

Note that the 50-60% estimate is proportional to **both** increases in SMRT Cell throughput (fast) and algorithm improvements (slow). This figure is likely to increase somewhat with successive chemistry releases.

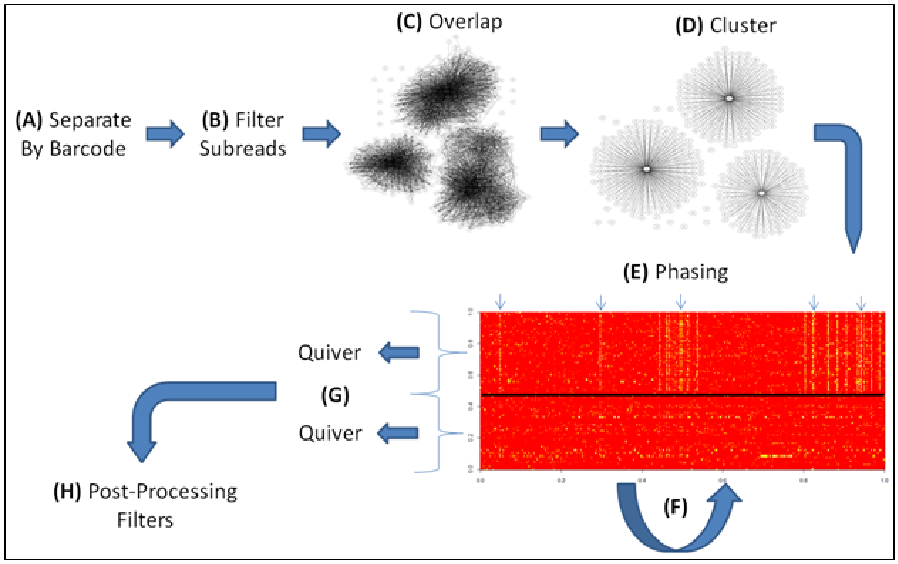
Long Amplicon Analysis: Software Overview

Pacific Biosciences' **Long Amplicon Analysis** protocol software:

- Generates *de novo* consensus sequences from pooled amplicon samples. Each amplicon can come from a diploid or polyploid organism; the software uses any differences between the alleles to split the consensus sequence into multiple haplotypes.
- Allows for accurate allelic phasing and variant calling in large genomic intervals.
- Supports the analysis of novel haplotypes in biomedical loci of interest, such as the HLA region in the human genome.
- Can pool up to **5** distinct amplicons. Reads are clustered into high-level groups, then each group is phased and consensus is called using the Quiver algorithm. Note that more amplicons may be pooled and clustered, however, the analysis time will be impacted.
- **Optionally** split reads by barcode if the sample is barcoded. The software uses the existing PacBio barcoding sample preparation and barcode-calling workflows. Reads are first split by barcode (**A**), then the reads for each barcode are processed independently (**B-H**).

The Long Amplicon Analysis software includes four main steps:

- **Coarse Clustering (C, D)**: Group reads from different amplicons into different clusters; detect read-read overlaps; build an overlap graph, then cluster the overlap graph to break the graph into the final clusters. The coarse clustering step is generally successful in separating HLA-A, B, and C genes into separate clusters. Note that (C) is the process of calculating the 'distance' between pairs of reads using a particular expression given overlap metrics. In step (D), the distances are entered into a matrix which then undergoes a Markov Clustering process (contraction/expansion iteration).
- **Phasing (E, F)**: Load the reads for each cluster into the Quiver consensus software and find an initial consensus. Recursively split reads from different haplotypes or other PCR products based on high scoring mutations proposed by Quiver. Note that (E) is the process by which each cluster is split into two haplotypes using a Quiver-like phasing process. This process is recursive (F): after each group is split (into 2) each of the resulting phases re-enter the algorithm to be potentially split again until no more (sub)groups are split.
- **Consensus (G)**: Generate a final consensus for each haplotype or PCR product using Quiver.
- **Post-Processing Filters (H)**: Detect and remove PCR artifacts. Chimeric sequences are identified using the UCHIME algorithm, and other PCR artifacts are identified by overall consensus quality.



Input Data

The **Long Amplicon Analysis** protocol software takes as input full-length amplicon sequences in the form of `*.bax.h5` files. The amplicons can be any length up to a small multiple of the polymerase read length.

Output Data

The **Long Amplicon Analysis** protocol software produces the following output files:

- `amplicon_analysis_chimeras_noise.fasta/fastq`
(Contains sequences flagged by the Chimera and noise filters.)
- `amplicon_analysis.csv`
(Contains 1 row per base, per FASTA sequence, including coverage and QV information. **Note**: QV scores are on a PHRED+33 scale.)
- `amplicon_analysis.fasta/fastq`
(Contains 1 line per barcode, per haplotype. Includes consensus sequences for all “True” alleles.)
- `amplicon_analysis_summary.csv`
(Contains 1 line per sequence; lists which filters were passed or failed.)
- `amplicon_analysis_subreads.csv`
(Contains 1 row per subread and 1 column per consensus sequence, mapping the contribution posterior probabilities of each subread to each consensus sequence.)

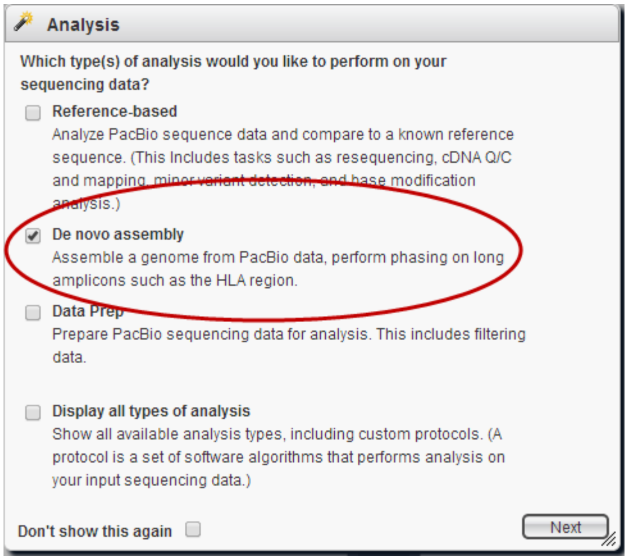
If a consensus sequence is **not** included in the `amplicon_analysis.fasta/q` files, it may have been incorrectly flagged as a Chimera or other PCR artifact and stored in the `amplicon_analysis_chimeras_noise.fasta/q` files.

If the consensus sequence is **not** in any of the `amplicon_analysis` files, it was **not** found by Long Amplicon Analysis.

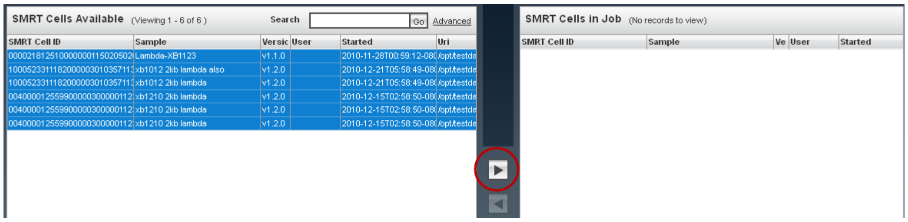
Running the Protocol Using SMRT® Portal

1. Log into SMRT Portal and click **Create New**.

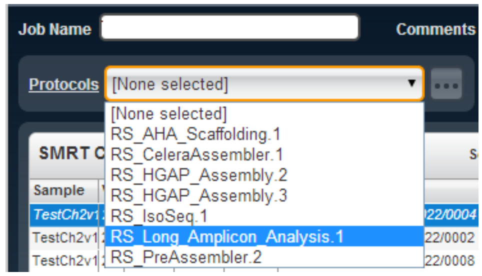
2. Check **De novo assembly** in the Analysis dialog, then click **Next**. **Note**: Depending on what the SMRT Portal Administrator specified, this dialog may **not** display.



3. Search for and select your data from the **SMRT Cells Available** Panel on the left. Click the **right-arrow** button to add the selected cells to the **SMRT Cells in Job** box. Data from these cells will be analyzed as part of the job.

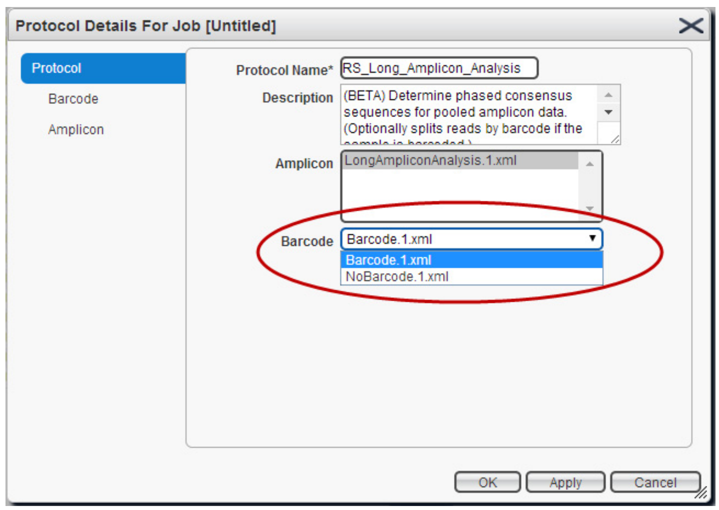


4. Select **RS_Long_Amplicon Analysis.1** from the **Protocols** list.

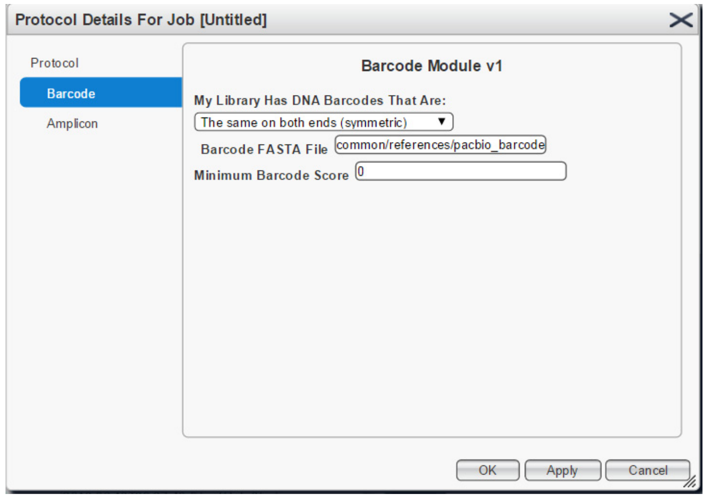


5. Click the "**...**" button to specify protocol settings.

6. (**Optional**) Select a barcode protocol if the sample is barcoded.



7. (**Optional**) Customize the barcode settings.



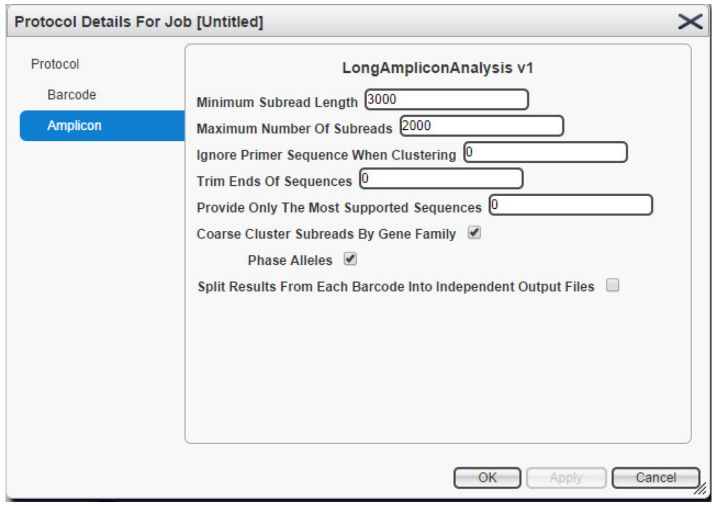
- **My Library has DNA Barcodes That Are:**
  - **Paired**: A pair of barcode sequences that always occur together, and are different on each end.
  - **Symmetric**: A single barcode sequence that occurs at both ends of the insert sequence.

- **Barcode FASTA FIle:**
  Specify the path to the FASTA file containing the barcodes to use.
- **Minimum Barcode Score** (`--minBarcodeScore`)**:**
  Subreads from ZMWs with barcode scores **below** this value are filtered out.

  Which cutoff value should you use? Empirical tests with symmetric 16 bp barcodes have ~99% accuracy with a minimum barcode score of **22**.

  In general, lower thresholds produce spurious consensus sequences which typically have low consensus accuracy and are removed by downstream filters. However, applying a barcode filter in the beginning stage improves overall performance when there are sufficient subreads at low to moderate multiplexing.

8. (**Optional**) Customize the amplicon settings.



- **Minimum Subread Length** (`--minLength`):
  Subreads shorter than this value are filtered out.

  Which cutoff value should you use? Even in experiments with the same-sized amplicons, you may require different settings depending on the quality of the PCR product.

  Higher thresholds produce consensus sequences with more consistent quality scores on the 5' and 3' ends and fewer truncations, at the cost of lower coverage. On a SMRT Cell containing only one sample, low coverage won't be an issue, but on multiplexed samples it can be an important concern.

  In general, the cutoff should **not be less** than ~80% nor greater than ~95% of the length of the minimum amplicon size. For the most common ~3.4-3.5 kb HLA Class I amplicons, we recommend a cutoff of between **3000 bp** and **3200 bp**.

- **Maximum Number of Subreads** (`--maxReads`):
  Specify the maximum number of subreads to use for read clustering and consensus.

  Which value should you use? For a sample containing **X** amplicons, assuming all amplicons are equally represented in the sample, we recommend that this be set to approximately **700 * X**. This uses enough reads such that the tool will have the 500 reads recommended for phasing and consensus with a safety margin, while being low enough to ensure a speedy completion time.

  We recommend **700** for a single amplicon and **2000** for a mixture of three amplicons. Samples showing large disparities between the prevalence of different amplicons, or high levels of off-target hits and PCR artifacts, require **higher** values.

- **Ignore Primer Sequences when Clustering** (`--ignoreEnds`): Specify the lengths of the primers to ignore when finely clustering subreads.

  This option reduces unnecessary splitting of haplotypes that commonly occurs when degenerate primers are used in PCR. If that applies to your experiment, the recommended value is:

  `Length of Barcodes (if any) + Length of Longest Primer + 5 (margin of error)`

  Or a value of **30** for non-barcoded samples with 25 bp primers, and **50** for padded, barcoded samples with the same primers.

- **Trim Ends of Sequences**: Specify the number of bases to trim from the ends of consensus sequences. This option is for removing primer sequences from allele consensus outputs, for proprietary primers. The recommended length is the same as the **Ignore Primer Sequences when Clustering** option.

- **Provide Only The Most Supported Sequences:** Specify the number of best-supported sequences to report from each coarse cluster. This option is useful for HLA users who want an extra layer of filtration to remove extra, often spurious, sequences per cluster. Generally for Class I alleles, each locus is represented by a single coarse cluster. Setting this value to `2` returns the two best sequences, representing the two haplotypes for each cluster. HLA Class II alleles are more frequently split across more than one cluster per locus due to greater differences between haplotypes, so we do **not** recommend using this option for Class II alleles. (`0` disables the filter.)

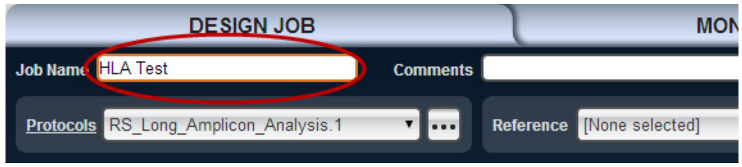
- **Coarse Cluster Subreads by Gene Family**: Specify whether or not to perform Markov clustering of subreads into rough gene families. If this is **not** set, all subreads are grouped into one gene family. **Uncheck** this option when analyzing single-locus, single amplicon samples, in which case the phasing step alone is desired.

- **Phase Alleles:** Specify whether or not to separate highly-similar alleles using phasing analysis. **Uncheck** this option to return only **one** consensus sequence per cluster.

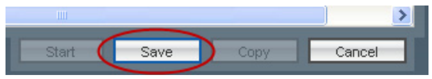
- **Split Results from Each Barcode into Independent Output Files**: Specify whether or not to split the results from each barcode into two separate zip files containing FASTA and FASTQ files. This option is useful if your downstream typing software/analysis pipelines requires separate output files for each barcode.

9. Click the **Apply** button at the bottom of the dialog.

10. Enter a name for the secondary analysis job.



11. Click **Save**, then click **Start**.



Note: To access the SMRT Portal help, click the **Help** link:

SMRT® Portal Reports

After the secondary analysis job is finished, SMRT Portal displays two reports.

The **Amplicon - Input Metrics** report displays statistics on the input amplicon sequences:

Amplicon Input Molecule Summary						
Sample	Good	Good (%)	Chimeric	Chimeric (%)	Noise	Noise (%)
0	1,450	100.0%	0	0.0%	0	0.0%
All	1,450	100.0%	0	0.0%	0	0.0%

- **Sample**: The number of the sample.
- **Good/Good (%)**: The number/percentage of ZMWs contributing to consensus sequences **not** categorized as Chimeric or Noise.
- **Chimeric/Chimeric (%)**: The number/percentage of ZMWs contributing to consensus sequences flagged as likely coming from PCR cross-over events.
- **Noise/Noise (%)**: The number/percentage of ZMWs contributing to consensus sequences that have a very low predicted accuracy (<95%) despite sufficient coverage (>20 reads and >10% all sequences in the current bin) to be called an novel allele.

The **Amplicon - Consensus Summary** report displays statistics on the amplicon sequences:

Amplicon Consensus Summary				
Sequence Cluster	Sequence Phase	Length (Bp)	Estimated Accuracy	Subreads Coverage
Cluster0	Phase0	3,427	99.994%	500
Cluster1	Phase0	3,404	99.994%	500
Cluster2	Phase0	3,444	99.994%	481

- **Sequence Cluster**: A name given to the cluster of sequences roughly corresponding to one amplicon.
- **Sequence Phase**: A name given to each phased haplotype within a sequence cluster.
- **Length (Bp):** The length of the consensus amplicon sequence.
- **Estimated Accuracy**: The estimated accuracy of the consensus amplicon sequence.
- **Subreads Coverage**: The number of subreads used to call consensus for this sequence.

How Long Will Long Amplicon Analysis Take?

For **one** sample:

- On a 16-CPU compute node, analysis takes approximately 12-15 minutes per sample, or approximately 3-4 minutes per locus plus 3-4 minutes for the initial clustering.

For a **multiplexed** run:

- On a 16-CPU compute node, analysis takes approximately 12-15 minutes per sample, or approximately ~8-10 hours for a run on 45x multiplexed samples.

Note: You do **not** need a high-performance compute cluster to use Long Amplicon Analysis. Pacific Biosciences provides a free Amazon Machine Image that you can use to run Long Amplicon Analysis in the cloud. See **http://www.pacb.com/devnet/** for details.

More detailed material on Long Amplicon Analysis is available on Pacific Biosciences' GitHub page. See **https://github.com/PacificBiosciences/SMRT-Analysis/wiki** for details.